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DOES EUTROPHICATION ENHANCE CD BIOAVAILABILITY BY
TROPIC TRANSFER TO THE MARINE AMPHIPOD *LEPTOCHEIRUS*
PLUMULOSUS FROM MICROALGAE?

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Biological Sciences

by

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ABSTRACT

Bioavailability and nutrient effects on the trophic transfer of Cadmium (Cd) associated with microalgae to the marine amphipod, *Leptocheirus plumulosus*, were investigated. Cd assimilation efficiencies (AE) were measured by a pulse-chase technique using a radiotracer. Cd AE in *L. plumulosus* significantly varied among algal species tested, and was highest (38.8 %) for the benthic diatom *Nitzschia punctata*, lowest (5.9%) for the planktonic diatom *Thalassiosira weissflogii*, and intermediate (15.6%) for the planktonic dinoflagellate *Isochrysis galbana*. Instantaneous egestion rates of Cd displayed a typical biphasic pattern over 96 h of depuration. Depuration in seawater-only of *L. plumulosus* yielded the highest Cd AE of 35.0%, whereas AEs in which depuration occurred in natural sediment and processed sediment were only 5.3% and 4.3%, respectively. Body size, ranging from 0.5 to 2.0 mm, of *L. plumulosus* feeding on labeled *T. weissflogii* did not affect Cd AEs.

Nitrate enrichment from 0-180 μM on algae significantly increased Cd AEs by *L. plumulosus* from 9.4-18.8% for *T. weissflogii*, from 10.0-27.3% for *N. punctata*, and from 10.0-16.2% for *I. galbana*. Physiological turnover (elimination) rate constants of Cd in *L. plumulosus* ranged from 0.016-0.025 h^{-1} for the three algal species, and were independent of nitrate addition. Algal fractionation revealed that nitrate enrichment strongly enhanced the fraction of Cd associated with cytoplasm, which probably contributed to the increased Cd AEs by *L. plumulosus*.

Phosphate addition (0 - 7.5 μM) on algae showed that Cd AEs of *L. plumulosus* were from 26.4-35.8% for *T. weissflogii*, and from 15.3-18.5% for *N. punctata*.

Phosphate enrichment did not significantly affect trophic transfer of Cd from algae to *L. plumulosus*. Cd fractionation in cytoplasm showed no obvious correlation with phosphate addition.

Overall, there was a significant linear relationship between the Cd AE of *L. plumulosus* and the distribution of Cd within algal cells. My work suggests that eutrophication by nitrate enrichment, but not phosphate, has the potential to enhance the trophic transfer of metals from pelagic and benthic microalgae to grazers in coastal benthic food webs.

INTRODUCTION

Coastal eutrophication has become a worldwide problem that is known to reduce water quality and impair aquatic ecosystem function. The annual nitrite load to the northern Gulf of Mexico continental shelf from the Mississippi River has almost doubled from the mid-1960s to the mid-1980s (Turner and Rabalais, 1991). In Louisiana, the escalating delivery of river-borne nutrients has dramatically increased deposition rates of biologically bound silica of diatoms onto shelf sediments, which potentially modifies coastal food webs, and has created extensive areas of bottom-water hypoxia (Turner and Rabalais, 1994). Nutrient enrichment in coastal ecosystems not only causes an increase in microalgal biomass but also has the potential to alter the bioavailability and fate of toxic metals (Skei et al., 2000; Schaanning et al., 1996).

Benthic and planktonic microalgae are generally considered to be the ultimate food sources for many marine invertebrates and fishes, and to occupy the base of the food chain in estuarine ecosystems (Sullivan and Moncreiff, 1990). For example, Pinckney et al. (2003) demonstrated that, in the upper few millimeters of saltmarsh sediments, there was a strong trophic relationship between benthic microalgae and meiofauna. Slightly higher than the Redfield ratio (106:16:1) typical of pelagic microalgae, an optimal stoichiometric ratio for benthic algae has been proposed to be 119:17:1 for C, N and P, respectively (Hillebrand and Sommer, 1999). However, under eutrophication, the nutrient N/P ratio in water column changes considerably (Colern, 2001; Rabalais et al., 1996) and these changes are known to alter microalgal communities and ecological relationships. For example, enrichment with NH_4^+ in saltmarsh sediments caused significant increases

in the primary productivity and standing crop of benthic microalgae (Darley et al., 1981). Nilsson et al.(1991) showed that nutrient enrichment to a sandy sediment increased microalgal biomass by a factor of 4 due to increases in diatoms and filamentous cyanobacteria, while bacterial productivity responded only weakly. After examining the benthic diatom flora of sediments near a sewage treatment plant, Agatz et al. (1999) concluded that microalgal biomass increased with increasing quantities of nutrients, and the diatom community varied with nutrient concentrations. Thus, eutrophication is typically expected to increase microalgal production and alter the structure of benthic microalgal communities. Increased levels of nutrients and metals are both becoming more common in many coastal areas including salt marshes. Few studies have examined the interaction between nutrient enrichment and the trophic transfer of metals in coastal benthic ecosystems. It is possible that eutrophication will cause increased biotic exposure to metals via benthic food webs. The net result might lead to changes in trophic transfer of contaminants from microalgae to grazing invertebrates.

Luoma et al. (1998) found that Cd concentration in phytoplankton increased by five fold during a spring phytoplankton bloom, presumably due to increased intracellular uptake of Cd by algae. Riedel and Sanders (2003) found that increased nutrients generally decreased dissolved trace element concentrations in the water column of estuarine microcosms, primarily through an increase in phytoplankton biomass, but also by increasing the concentration of metals in the seston. By examining planktonic algae, nutrient enrichment was found to significantly increase the uptake of Cd and Zn in both marine and freshwater algae (Wang et al., 2001a, b; Yu and Wang, 2004a). By further providing labeled algae to grazers, nitrogen enrichment for marine algae significantly

increased assimilation efficiencies (AE) of Cd and Zn to pelagic copepods (Wang et al., 2001a, b). However, no significant effects were found for AEs of Cd and Zn in *Daphnia* after feeding freshwater algae under conditions of nutrient enrichment (Yu and Wang, 2004b). It is hypothesized that the concentration of cellular protein ligands for Cd binding, transport and sequestration in marine algae increased with increasing nitrate levels, leading to an enhanced Cd uptake into algal cells. This suggests that nutrient enrichment may lead to increased metal uptake by grazing invertebrates, possibly due to an alteration in the distribution of metals in algal cells. Schaanning et al. (1996) stated that the bioaccumulation of Hg and Cd in three species of infaunal macroinvertebrates (*Abra alba*, *Nereis diversicolor* and *Amphiura filiformis*) was significantly stimulated by adding algal detritus to a suspension of seawater and sediment. Whether nutrient enrichment by nitrate or phosphate has effects on the uptake and trophic transfer of metals from planktonic and benthic microalgae to benthic animals remains largely unknown, and it is important to identify potential interaction mechanisms between eutrophication and contaminants. Benthic invertebrates are known to consume a large fraction of settled phytoplankton and benthic microalgae (Sullivan and Moncreiff, 1990; Pinckney et al., 2003) and play important roles in energy and contaminant transfers to higher trophic levels. Furthermore, sediments are frequently contaminated with metals and metal pollution is long-lived in sediments.

The goal of the study was to investigate how nutrient enrichment affects the bioavailability and trophic transfer of Cd associated with microalgae to the benthic estuarine amphipod *Leptocheirus plumulosus* Shoemaker. *L. plumulosus* is a euryhaline (e.g., 0-32 ppt) and infaunal amphipod broadly distributed along the east coast of North

America (Bousefield, 1973; Schlek et al., 1992). This marine amphipod utilizes both suspension feeding of suspended particles (e.g., algae) and surface deposit feeding on sediment and detritus particles including phytodetritus (Dewitt et al., 1992). In benthic food chains, *L. plumulosus* serves as an important prey to fish and shellfish in nature (Hines et al., 1990). *L. plumulosus* is also a sensitive species commonly used in toxicity tests of sediment (Schlek et al., 1992; McGee et al., 1993; US EPA, 1994a) and dredged material (US EPA, 1994b; US EPA and US ACE, 1998). It is generally assumed that the heavy metal Cd, used in these experiments, is a nonessential element in invertebrates but may serve as a nutrient substitute for Zn in marine diatoms (Price and Morel, 1990). The trophic transfer of Cd was quantified by estimating metal assimilation efficiency (AE) in *L. plumulosus*, which is an important parameter quantifying metal bioavailability from dietary uptake (Wang and Fisher, 1999; Schlek et al., 2000). The null hypothesis of the studies was that nutrient enrichment does not influence the assimilation efficiencies of Cd associated with benthic and pelagic microalgae by *L. plumulosus*.

MATERIALS AND METHODS

Three marine microalgal species, the benthic diatom *Nitzschia punctata* (CCMP561), the planktonic diatom *Thalassiosira weissflogii* (CCMP1050), and the planktonic dinoflagellate *Isochrysis aff. galbana*, were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton, West Boothbay Harbor, ME. These three species are abundant in coastal or estuarine areas and are available to be the diet of benthic amphipods. Algal stocks were maintained under axenic conditions in an f/2 medium (Guillard and Ryther, 1962) at 19 °C and under light illumination of 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ with a 14:10 h light: dark cycle. Artificial seawater (20‰) used in all experiments was prepared with commercial sea salts (Crystal Sea[®]) diluted with deionized water and filtered through 0.45 μm polycarbonate membranes. The estuarine amphipod, *Leptocheirus plumulosus* Shoemaker, originally obtained from U.S. Army Corps of Engineers, Waterways Experiment Station, MS, has been cultured in our laboratory for 2 years following standard methods (U.S. EPA, 1994a).

Adult amphipods between 1.0-2.0 mm in length were collected for the experiments (except the body size experiment) by pouring culture sediment through a sieve series including 2.0 and 1.0 mm mesh screens. The original sediment for culture was taken from Terrebonne Bay estuary (29° 13' N, 90° 38' W) near the Louisiana Universities Marine Consortium (LUMCON) laboratory in Cocodrie, LA. Raw sediment was composed primarily of silts (41%) and clays (17%), and had an organic content of ca. 2.5% (Chandler & Fleeger, 1983). Healthy animal cultures were maintained at 23 °C

with sediment that was processed by the methods of Chandler (1986) (<212 μm , flushed, and sterilized).

Cd Assimilation Efficiency (AE)

AEs of Cd in *L. plumulosus* from microalgae were measured by using a pulse-chase technique (Wang et al., 1996; Schlegel et al., 2000). Our preliminary experiments indicated that burrowed amphipods seldom acquired enough radioactivity to adequately detect the overall kinetic depuration process when they were fed labeled natural surface sediment or microalgae. In addition, high variation also occurred among replicates and an even distribution of labeled microalgae in the feeding chamber with sediment was hard to create for the pulse-chase feeding for burrowed *L. plumulosus*. Generally, metal AEs increase significantly with decreasing algal food concentrations (Xu and Wang, 2001; Yu and Wang, 2002). Because *L. plumulosus* readily tolerates aqueous-only exposure for extended periods if food is provided (Dewitt et al., 1992), the pulse-chase feeding in this study was therefore conducted without sediment. After acclimation for 12 h in seawater, feeding activity and other behaviors of *L. plumulosus* were not obviously affected. The overall mortality rate of animals in 96 h depuration period for each beaker was less than 5% (Yu, pers. obs.). However, whether water-only conditions may affect feeding and other behaviors of the animals was not determined in these experiments.

The procedures for Cd AE measurements in *L. plumulosus* are found in Figure 1. Algal cells in the late exponential growth phase were collected onto 1- μm polycarbonate membranes and resuspended in f/2 medium (including 882.4 μM for NaNO_3 , 36.2 μM for NaH_2PO_4) prepared with 0.45- μm -filtered artificial seawater without the addition of Cu, Zn and Na_2EDTA . The initial cell concentrations in the medium were generally 7.0×10^5

cells ml^{-1} for *N. punctata*, 1.5×10^5 cells ml^{-1} for *T. weissflogii*, and 1.5×10^6 cells ml^{-1} for *I. galbana*. Radiotracer ^{109}Cd (in 0.1 N HCl) was obtained from PerkinElmer Life and Analytical Sciences, Boston, MA. Radioisotope additions to the culture media were 555 kBq L^{-1} (corresponding to 36.7 nM or $4.12 \mu\text{g L}^{-1}$) for ^{109}Cd in acid-cleaned polycarbonate flasks. Spiked Cd concentrations due to radioisotope addition were comparable to or lower than the background metal concentrations in most estuarine and coastal regions (Wang and Dei, 2001a) and far lower than the contamination levels in many coastal sediments (e.g., Cd, $0.5 \mu\text{g/g}$; Summers et al., 1996). A microliter volume of 1.0 N Suprapur NaOH was added into the $0.45 \mu\text{m}$ filtered seawater to maintain the pH at 8.1.

After 5-6 d, the algal cells had performed 3-5 divisions and were considered uniformly labeled. The cells were then collected by filtration onto $1 \mu\text{m}$ polycarbonate membranes, rinsed several times with $0.45 \mu\text{m}$ -filtered unlabeled seawater to remove radioisotopes weakly bound to the cell surface, and resuspended in filtered seawater. Cell densities were counted with a hemocytometer prior to feeding experiments.

One day before AE experiments, adult amphipods retrieved from culture sediment by sieves (1 and 2 mm mesh) were fed with one of the three mono-specific algal diets in seawater, allowing animals to acclimate to water-only environments and allowing their digestive system to acclimate that diet. *L. plumulosus* with similar body length (1-1.5 mm) were selected and allowed to evacuate their guts for 5-6 h in the test chambers (plastic beakers, 150 ml) with no food. Radio-labeled algae of the same species were then added separately into the feeding chambers containing 100 ml of filtered seawater and 10 adult amphipods. Water was then gently stirred by hands to keep algae in suspension. In

all experiments, feeding densities of algal cells were 2×10^4 to 1×10^5 cells ml^{-1} for *T. weissflogii*, 2×10^5 cells ml^{-1} for *N. punctata*, and 7×10^4 to 2×10^5 cells ml^{-1} for *I. galbana*.

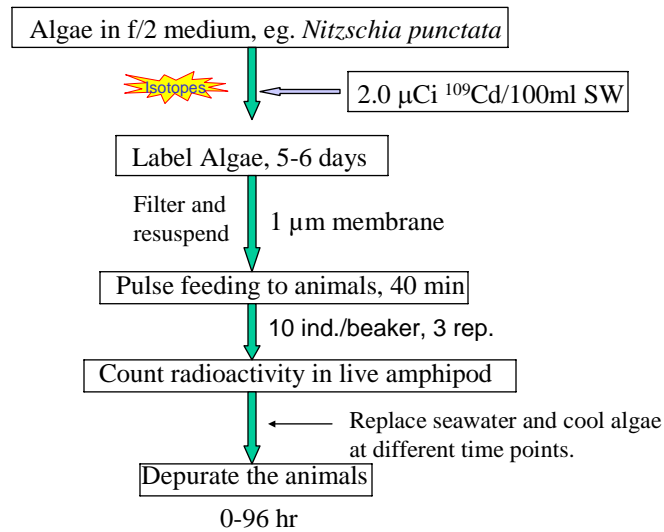


Figure 1. The diagram of flow chart of Cd assimilation efficiency measurements in *L. plumulosus*.

Three replicates were used for each treatment. The duration of the radioactive feeding time was 40 min, which was shorter than the gut passage time of *L. plumulosus*, in order to minimize voiding of radioactive feces. Mean gut passage time for *L. plumulosus* was previously found to be 95 min (Schlekat et al., 1999). In seawater-only conditions, mean gut passage time for the amphipods was 65 min for *T. weissflogii* at a cell density of 1×10^5 cells ml^{-1} , 70 min for *N. punctata* at a cell density of 2×10^5 cells ml^{-1} , and 90 min for *I. galbana* at a cell density of 2×10^5 cells ml^{-1} (Yu, pers. obs.). Gut passage time was

cell-density dependent. Feces generally appeared as the shape of tight floc or rod and were easily collected (Yu, pers. obs.). After pulse feeding, amphipods were collected on a 500 μm sieve, rinsed with non-radioactive water, and immediately placed in counting tubes with filtered water for radioactivity measurements. *L. plumulosus* was then placed in 100 ml of filtered seawater to eliminate the ingested radio-labeled food under the same conditions and in the presence of non-radioactive algae cultured under the same nutrient regimes. Radioactivity in the amphipods was repeatedly measured over the 96 h depuration period at time regular intervals (every 4-24h). The seawater and algal diet were replaced each time radioactivity was counted.

Fecal pellets egested during the radioactive feeding period and the depuration period were immediately collected on a 45 μm mesh, rinsed with filtered seawater, and assayed for radioactivity. At the beginning and end of the pulse-feeding period, the partitioning of trace metal Cd in radio-labeled algae was monitored by filtering a 10 ml feeding suspension onto a 1 μm polycarbonate membrane. In all experiments, 85-100% of Cd were associated with the radio-labeled particles by examining the distribution of radiotracer Cd in the filtered algae over that in the whole suspension.

The total amount of radioactivity ingested by *L. plumulosus* during the radioactive feeding period was calculated as the sum of the radioactivity in amphipod bodies and the radioactivity in the feces collected after 40 min of feeding on radioactive material. Cd assimilation efficiency (AE) was calculated as the intercept of the linear regression between the natural log of the percentage of Cd retained in animals and the time of depuration during the second compartment of elimination (24-96 h) (Wang and Fisher, 1999):

$$\%A = A_0 e^{-kt},$$

where %A is the percentage of radioactivity retained in *L. plumulosus* at time *t* in 4 d of metal elimination. *A*₀ is the intercept at y-axis (% retained radioactivity in the animals) and can be determined as the Cd AE. *k* is the physiological turnover rate constant (or the elimination rate constant) in the 4-d period and can be calculated as the slope of the linear regression between the natural log of the percentage of Cd retained in the animals and time of second elimination phase. *t* is time in hours. Biological half-lives of metals (*t*_{1/2}, d) in animal bodies was determined by the equation:

$$t_{1/2} = 0.693/k,$$

where *k* is the physiological turnover rate constant.

Preliminary experiments were performed to investigate the influences of algal species, substrate, and body size on Cd AE in *L. plumulosus*. For algal species experiments, three algae species were labeled and fed to amphipods. Instantaneous egestion rates of radioactive Cd were also estimated. Three substrates including seawater-only, natural sediment and processed sediment (Chandler, 1986) were employed in the 96-h elimination period to test the effects on the AE and release of Cd. For tests on amphipod body size, three size groups including 0.5-1.0mm, 1.0-1.5 mm and 1.5-2.0 mm (3 replicates for each group), with body dry weight of 447.0±9.4, 1635.2±109.8 and 2806.0±152.1 µg, respectively, were examined. In each treatment, there were 10-30 individuals for each replicate.

Nutrient Enrichment Effects on Cd Trophic Transfer

Microalgae were maintained in a semi-continuous culture. Experiments were designed to compare effects on the metal assimilation and depuration of *L. plumulosus*

feeding on algae grown under enrichment of different concentrations of nitrate (NO_3) (*T. weissflogii*, *N. punctata* and *I. galbana*) or phosphate (PO_4) (*T. weissflogii* and *N. punctata*). Algal species were presented in separate trials. Cells in the late log growth phases from the same batch were filtered and resuspended into 0.45 μm -filtered seawater containing different N (0, 60, and 180 μM) or P concentrations (0, 2.5 and 7.5 μM) at a cell density of 7.0×10^5 cells ml^{-1} for *N. punctata*, 1.5×10^5 cells ml^{-1} for *T. weissflogii*, and 6×10^5 cells ml^{-1} for *I. galbana* in acid-cleaned polycarbonate flasks. One replicate for algal labeling was used. The concentration range of nitrate and phosphate used were at environmentally realistic levels (Cloern, 2001). Other nutrients were maintained at the f/2 levels without addition of Cu, Zn and Na_2EDTA . Radiotracer ^{109}Cd was added to the culture media at microliter amount of 555 kBq L^{-1} (corresponding to 36.7 nM or 4.12 $\mu\text{g L}^{-1}$). When the cells reached the mid-exponential growth phase (1-2 days), they were transferred to a new medium containing the same concentration of N/P and radioactive ^{109}Cd . After 3-4 transfers (6-7 days), the cells were filtered again and resuspended in filtered seawater. Meanwhile, another set of algal cultures under the same nutrient conditions without presence of radiotracer ^{109}Cd were used for non-radioactive feeding during the depuration period. The same methods for pulse-chase feeding, AE determination and elimination were employed as described above.

The distribution of radioisotope in algal cells in the nutrient experiments was measured using a method modified from Fisher et al. (1983) and Reinfelder & Fisher (1991). Weakly surface bound metal was removed by a 1mM EDTA rinse. The cells were broken by suspension in Nanopure water and frozen at -20°C overnight. Thawed cells were further separated by centrifugation to produce three fractions including cell walls (at

2000 × g for 15 min), supernatant as cytoplasm and organelles (at 10,000 × g for 15 min). Cd removed by EDTA was added to the cell wall fraction to calculate the distribution of Cd.

Radioactivity Counting and Statistical Analysis

Radioactivity was determined using a Wallac 1470 Wizard gamma detector (Turku, Finland). Counting efficiencies were measured with appropriate standards and were calibrated for spillover and radioisotope decay. The gamma emissions of ^{109}Cd were detected at 88 keV. Counting times were 2 min and were sufficient to yield propagated counting errors <3%.

All data of assimilation efficiencies and physiological turnover rate constants were calculated by linear regression after the original percentage data of retained metal in animals were transformed in natural log. The data were first tested for normality. One-way ANOVA was used to compare the overall variation of analysis of the data. The specific comparisons among different treatments were performed by Tukey's honest significant difference (HSD) test as a post-ANOVA technique by SAS.

RESULTS

Cd Assimilation Influenced by Diet Species, Depuration Substrates and Body Size

Generally, *Leptocheirus plumulosus* displayed two phases for Cd elimination; the first characterized as an initial rapid loss (0-24 h), and the second as a slower release (24-96 h) (Figure 2). Instantaneous Cd egestion rate measurements (Figure 3) showed that most unassimilated Cd was egested within 24 h. After 24 h, egestion of Cd by *L. plumulosus* represented only a small fraction (<1%) of the total radioactive Cd in feces. Assimilation efficiency was therefore calculated as the y-intercept of the second slower compartment (24-96 h), assuming that *L. plumulosus* completed Cd digestion within 24 h and any loss of metal afterwards was due to physiological turnover. Results showed that the assimilation efficiencies of Cd by *L. plumulosus* were 5.9% for *Thalassiosira weissflogii*, 38.8% for *Nitzschia punctata*, and 15.6% for *Isochrysis galbana*, respectively. Cd AE for all the three species significantly differed from each other (One-way ANOVA and Tukey's test, $F=25.54$, $p=0.02$).

The physiological turnover rate constant (k) of ingested Cd was calculated as the slope of the linear regression between the natural log of the percentage of metal retained in amphipods and the time of depuration between 24 and 96 h, representing the loss rate constant from newly incorporated tissues after assimilation. The linear regression was significant for all treatments ($p<0.05$, $r^2 = 0.60-0.99$). Among the three species, Cd in the planktonic diatom *T. weissflogii* turned over at the highest rates, with a rate constant of 0.019 h^{-1} , followed by the planktonic dinoflagellate *I. galbana* (with the physiological turnover rate constant of 0.015 h^{-1}). ^{109}Cd from benthic diatom *N. punctata* was released

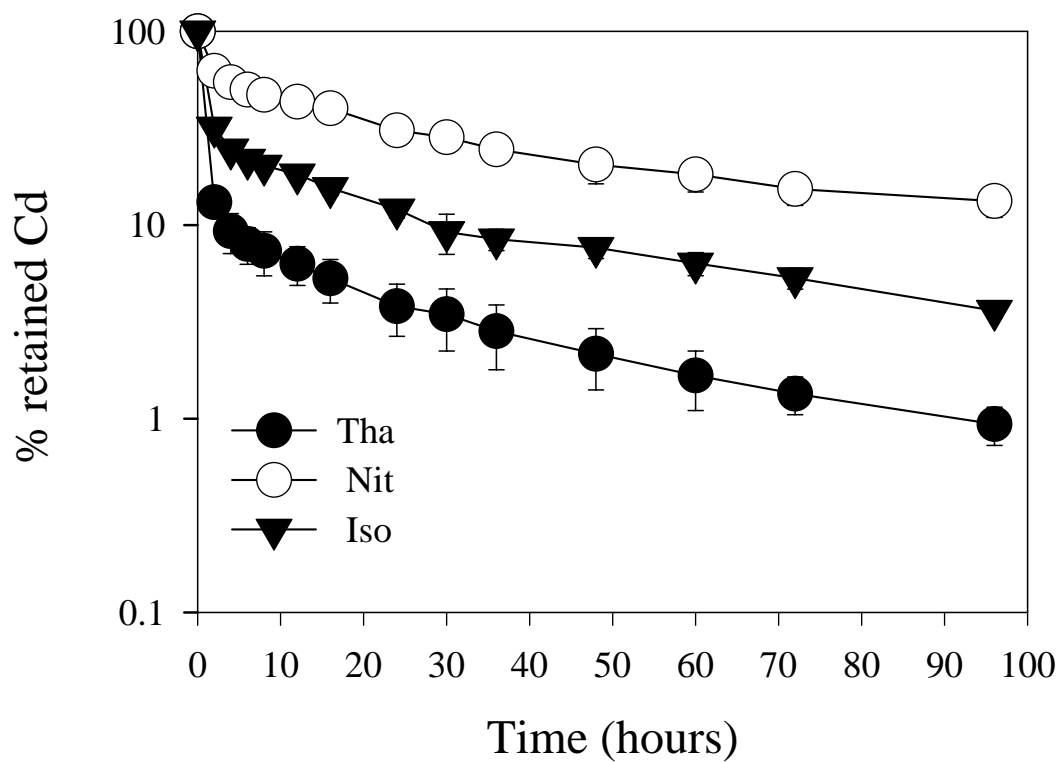


Figure 2. The retention of ingested Cd in *Leptocheirus plumulosus* after feeding on radio-labeled *Thalassiosira weissflogii*, *Nitzschia punctata*, and *Isochrysis galbana* grown in f/2 medium. Data points represent means and standard errors.

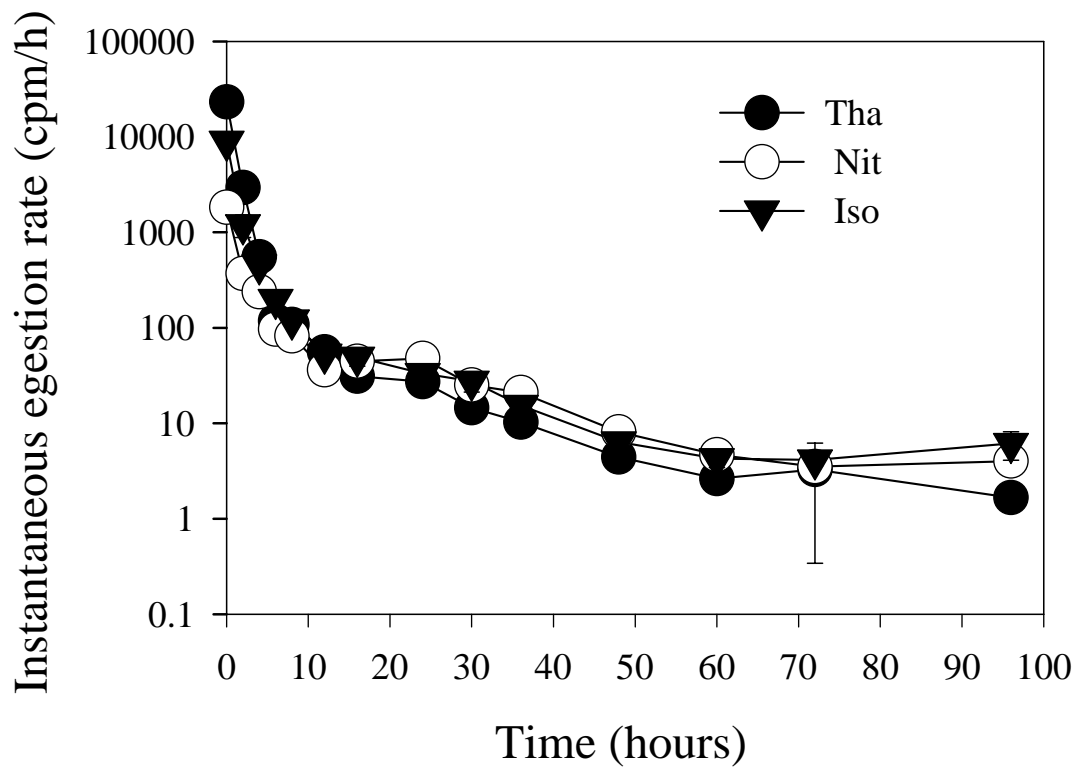


Figure 3. The instantaneous egestion rate (radioactivity egested per unit of time) of Cd in *Leptocheirus plumulosus* after feeding on radio-labeled *Thalassiosira weissflogii*, *Nitzschia punctata*, and *Isochrysis galbana* grown in f/2 medium. Data points represent means and standard errors.

at the slowest rate (0.012 h^{-1}). The biological retention half lives ($t_{1/2}$) of Cd were 57.7 for *N. punctata*, 47.2 for *I. galbana*, and 36.2 h for *T. weissflogii*, respectively. Algal species significantly affected the physiological turnover rate constants (One-way ANOVA, $F=12.71$, $p<0.05$) and the biological retention half lives of Cd in *L. plumulosus* ($F=20.86$, $p<0.01$). Tukey tests indicated that the values of k and $t_{1/2}$ for all three species differed significantly from each other.

After pulse-feeding radioactive algae to *L. plumulosus*, Cd depuration was evaluated for 96-h in 3 different substrates; seawater-only, natural sediment and processed sediment (Figure 4). Depuration in seawater-only yielded the highest Cd AE of 35.0%, whereas AEs in which depuration occurred in natural sediment and processed sediment were only 5.3% and 4.3, respectively. There was significantly higher Cd AEs when depuration occurred in seawater-only than in natural or processed sediment treatments (One-way ANOVA, $F=175.12$, $p<0.01$). No statistically significant variation appeared between the treatments in the natural sediment and in processed sediment (Tukey test). The physiological turnover rate constants (k) among the three substrates were comparable. The k values were 0.016, 0.017, and 0.015 h^{-1} for the seawater-only, natural sediment and processed sediment, respectively. Values were not significantly different (One-way ANOVA and Tukey test, $F=0.92$, $p=0.465$). The corresponding retention half lives ($t_{1/2}$) were 43.3 for the seawater-only, 42.4 for natural sediment, and 47.2 h for processed sediment. No significant effects were found for $t_{1/2}$ values among the three species ($F=0.79$, $p=0.510$).

Body size of *L. plumulosus*, fed labeled *T. weissflogii*, did not influence Cd AEs (One-way ANOVA and Tukey test, $F=8.15$, $p>0.081$) (Figure 5). Cd AEs were 28.5% for

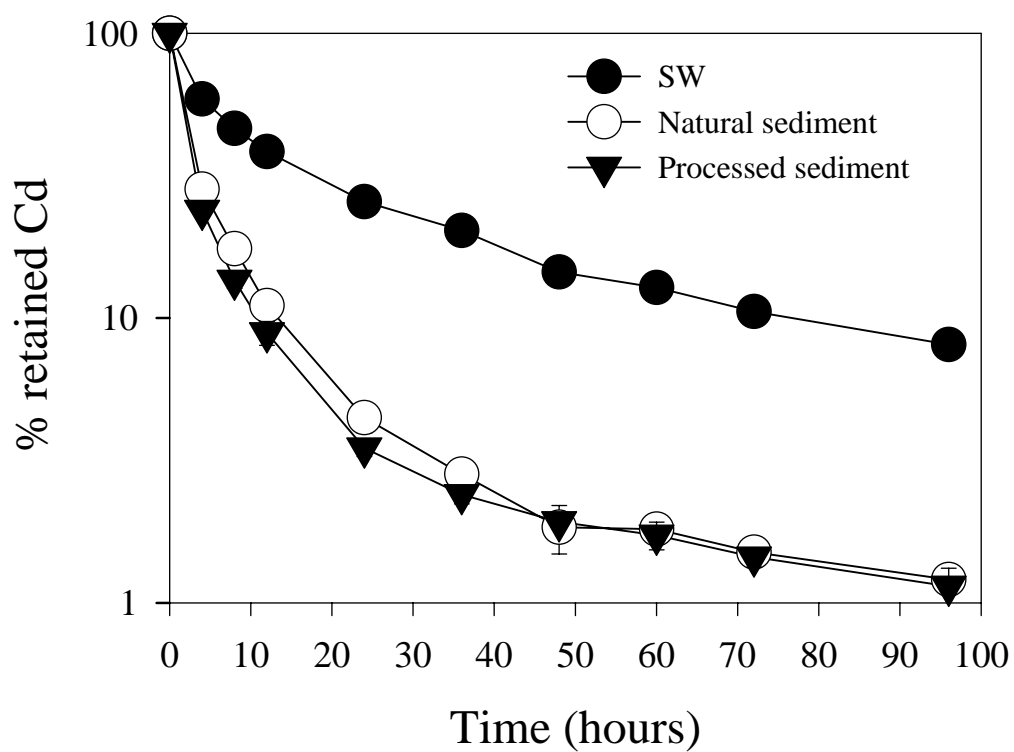


Figure 4. Depuration of ingested Cd by *Leptocheirus plumulosus* in different substrates after feeding on radiolabeled *Thalassiosira weissflogii* grown in f/2 medium. (SW): Seawater; (Natural sediment) Natural sediment from salt marsh; (Processed sediment): Processed sediment from natural salt marsh. Data points represent means and standard errors.

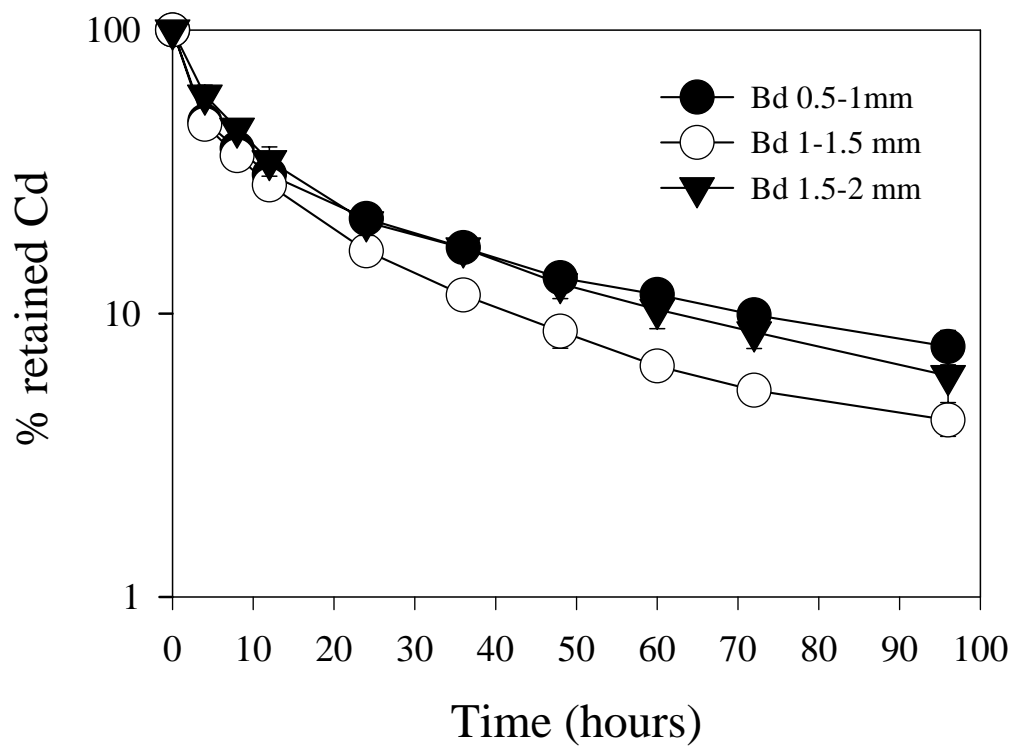


Figure 5. The retention of ingested Cd in *Leptocheirus plumulosus* with different body sizes after feeding on radio-labeled *Thalassiosira weissflogii* grown in f/2 medium. (Bd 0.5-1mm): Body size ranging from 0.5 to 1.0 mm; (Bd 1-1.5 mm): Body size from 1.0 to 1.5 mm; (Bd 1.5-2mm): Body size from 1.5 to 2.0 mm. Data points represent means and standard errors.

amphipods from 0.5-1.0 mm (Bd 0.5-1 mm, body size of 0.5-1 mm), 23.3% for the group from 1.0-1.5 mm (Bd 1-1.5 mm, body size of 1-1.5 mm), and 31.3% for the group from 1.5-2.0 mm (Bd 1.5-2.0 mm, body size of 1.5-2.0 mm). No significant effects of body size on the physiological turnover and biological retention half lives ($t_{1/2}$) of Cd were observed (ANOVA and Tukey test, $p > 0.05$). The physiological turnover rate constants were 0.014 in Bd 0.5-1 mm, 0.019 in Bd 1.0-1.5 mm, and 0.018 h⁻¹ in Bd 1.5-2.0 mm. The $t_{1/2}$ values were 48.6, 36.2, and 41.1 h for the groups of Bd 0.5-1 mm, Bd 1.0-1.5 mm, and Bd 1.5-2.0 mm, respectively.

Nitrate Enrichment Effects on Cd Trophic Transfer

The effects of elevated nitrate on trophic transfer of Cd in *L. plumulosus* from microalgae were quantified by measurement of Cd AE. Three species, *T. weissflogii*, *N. punctata*, and *I. galbana*, were separately employed to test nutrient enrichment effects. Similar depuration patterns with an initial fast egestion phase (within 24 h) followed by a slower elimination phase (24-96 h) were found in *L. plumulosus* (Figure 6).

Increased nitrate concentrations from 0-180 μ M significantly increased Cd AE by *L. plumulosus* in all three algal species (One-way ANOVA, $p < 0.05$; see Table 1). For *T. weissflogii*, the calculated Cd AEs were 2.0 \times higher in the amphipods fed with the diatom cultured at 180 μ M than in those fed with the same species cultured at 0 μ M. Distribution of Cd in the diatom cytoplasm was also found to be 1.5 \times higher in algae inoculated at 180 μ M than algae inoculated at 0 μ M. Statistical analysis exhibited that Cd AEs at 180 μ M were significantly higher than those at 0 and 60 μ M ($p < 0.05$, one-way ANOVA). No significant variation occurred between the Cd AE at 0 μ M treatment and that at 60 μ M

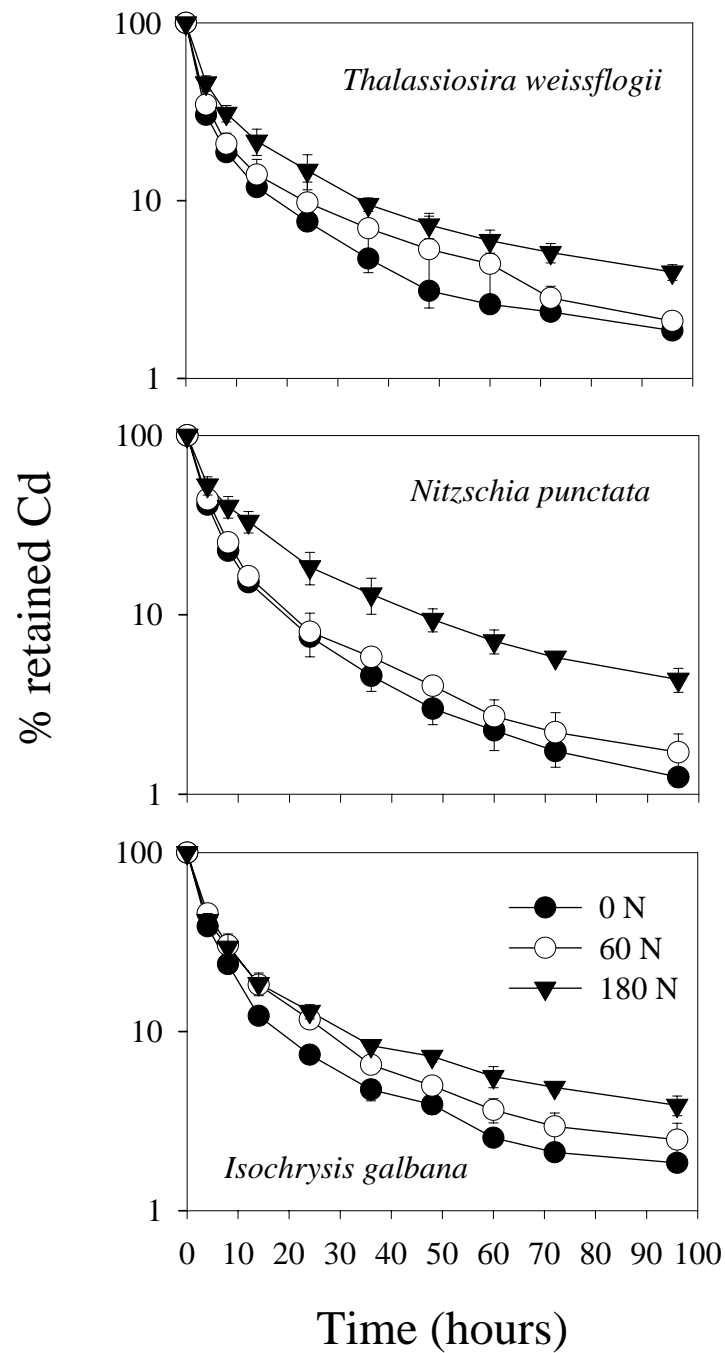


Figure 6. The retention of ingested Cd in *Leptocheirus plumulosus* after feeding on radio-labeled *Thalassiosira weissflogii*, *Nitzschia punctata*, and *Isochrysis galbana* grown under different nitrate concentrations. (0N): 0 uM; (60N): 60 uM; (180N): 180 uM. Data points represent means and standard errors.

Table 1. The assimilation efficiency (AE, %), the physiological turnover rate constants k (h^{-1}) and the biological retention half lives $t_{1/2}$ (hours) of Cd in *Leptocheirus plumulosus* feeding on microalgae under different nitrate treatments. Mean \pm SE (n=3). Data were compared among treatments using a one-way ANOVA followed by Tukey's honest significant difference (HSD) test ($p < 0.05$).

Algal N treatments ($\mu\text{mol L}^{-1}$)	AE (%)	Cd in cytoplasm (%)	k (h^{-1})	$t_{1/2}$ (hours)
<i>T. weissflogii</i>				
0	9.4 \pm 0.5 ^A	13.4 \pm 0.7 ^A	-0.019 \pm 0.002	37.2 \pm 2.5
60	9.5 \pm 0.3 ^A	17.1 \pm 0.9 ^{A, B}	-0.017 \pm 0.001	35.2 \pm 7.1
180	18.8 \pm 3.7 ^B	19.6 \pm 0.2 ^B	-0.017 \pm 0.001	40.1 \pm 2.3
<i>N. punctata</i>				
0	10.0 \pm 1.1 ^A	nd*	-0.020 \pm 0.003	35.3 \pm 5.2
60	11.3 \pm 2.0 ^{A, B}	nd*	-0.025 \pm 0.001	28.1 \pm 0.5
180	27.3 \pm 5.4 ^B	nd*	-0.020 \pm 0.004	36.6 \pm 9.0
<i>I. galbana</i>				
0	10.0 \pm 1.6 ^A	8.6 \pm 0.2 ^A	-0.020 \pm 0.002	35.8 \pm 2.9
60	15.3 \pm 2.4 ^{A, B}	11.7 \pm 0.6 ^{A, B}	-0.021 \pm 0.004	33.9 \pm 6.1
180	16.2 \pm 0.8 ^B	14.9 \pm 0.7 ^B	-0.016 \pm 0.001	43.1 \pm 1.6

* not determined

(Tukey test). For *N. punctata*, Cd AEs significantly increased 2.7× when the nitrate levels of algal culture increased from 0 to 180 μM ($p < 0.05$). However, the Cd AEs at 60 μM was comparable to those at 0 μM (Tukey test). For *I. galbana*, nitrate enrichment from 0-180 μM in algae also caused a significant increase of Cd AE from 10.0-16.2% ($p < 0.05$). A statistical difference occurred between the Cd AE of 0 μM nitrate treatment and that of 180 μM nitrate treatment ($p < 0.05$), but no apparent variation was observed between Cd AE of 60 μM nitrate treatment and that of 180 μM treatment (Tukey test). Cytoplasmic Cd partition increased by 1.7 × in *I. galbana* when nitrate concentration in the algae increased from 0-180 μM .

Physiological turnover rate constants (k) of Cd in *L. plumulosus* were independent of nitrate addition for the three algal species tested. The values of k were comparable for different nutrient status, ranging from 0.017-0.019 for *T. weissflogii*, 0.020-0.025 for *N. punctata*, and 0.016-0.021 h^{-1} for *I. galbana* (Table 1). Nitrate enrichment also did not cause significant influence on the biological retention half lives ($t_{1/2}$) of Cd (ANOVA and Tukey test). The general range of $t_{1/2}$ values for the three algae was from 28.1-43.1 h.

Phosphate Enrichment Effects on Cd Trophic Transfer

Phosphate enrichment (0, 2.5 and 7.5 μM) to radio-labeled algae did not significantly affect Cd assimilation efficiencies in *L. plumulosus* for *T. weissflogii* or *N. punctata* (one-way ANOVA and Tukey test, $p > 0.05$) (Figure 7), but did cause significant effects on Cd distribution in the cytoplasm of *T. weissflogii*. Cd AEs ranged from 26.4-35.8% for *T. weissflogii*, and from 15.3-18.5% for *N. punctata* (Table 2). The physiological turnover rate constant and the biological retention half lives were independent of the phosphate concentrations for both algal species.

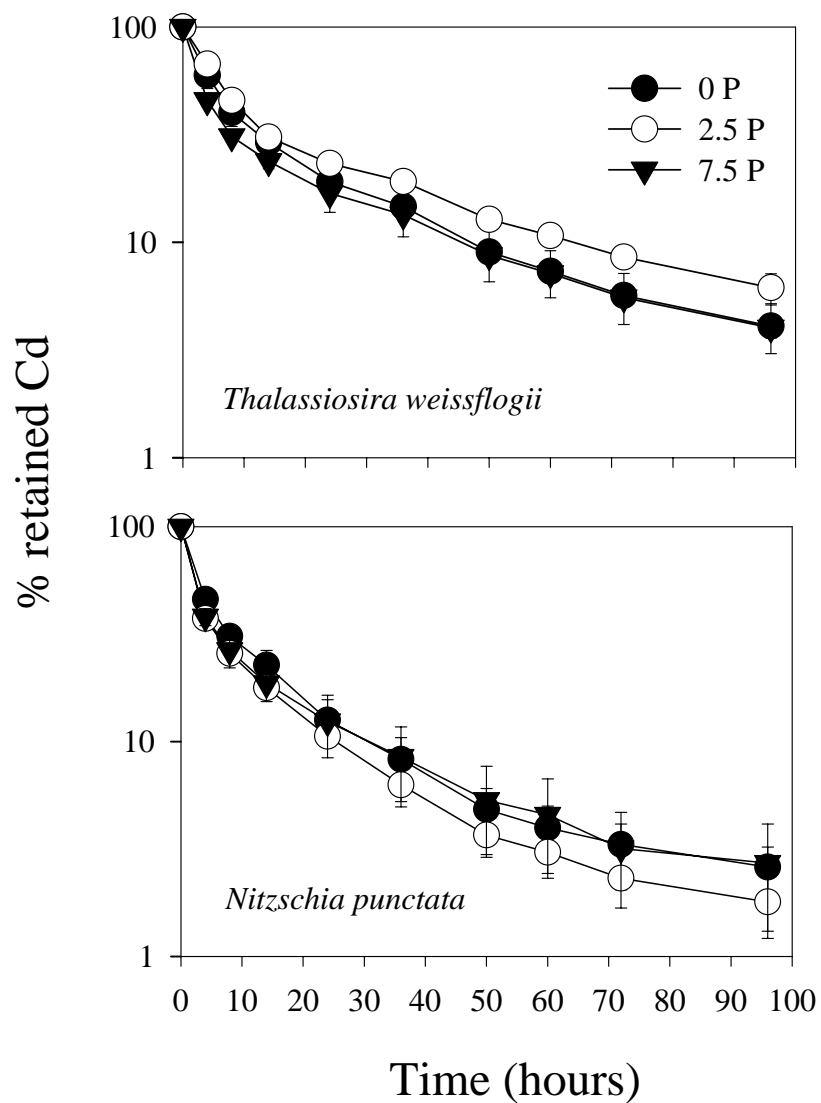


Figure 7. The retention of ingested Cd in *Leptocheirus plumulosus* after feeding on radio-labeled *Thalassiosira weissflogii* and *Nitzschia punctata* grown under different phosphate concentrations. (0 P): 0 uM; (2.5 P): 2.5 uM; (7.5 P): 7.5 uM. Data points represent means and standard errors.

Table 2. The assimilation efficiency (AE, %), the physiological turnover rate constants k (h^{-1}) and the biological retention half lives $t_{1/2}$ (hours) of Cd in *Leptocheirus plumulosus* after feeding on radiolabeled microalgae under different phosphate treatments. Mean \pm SE (n=3). Data were compared among treatments using a one-way ANOVA followed by Tukey's honest significant difference (HSD) test ($p<0.05$).

Algal P treatments ($\mu\text{mol L}^{-1}$)	AE (%)	Cd in cytoplasm (%)	k (h^{-1})	$t_{1/2}$ (hours)
<i>T. weissflogii</i>				
0	30.2 \pm 8.6	37.9 \pm 0.1 ^A	-0.022 \pm 0.001	31.6 \pm 0.8
2.5	35.8 \pm 3.0	29.5 \pm 0.8 ^{A, B}	-0.019 \pm 0.002	36.6 \pm 4.3
7.5	26.4 \pm 2.0	28.7 \pm 1.6 ^B	-0.021 \pm 0.002	33.7 \pm 2.5
<i>N. punctata</i>				
0	17.5 \pm 4.7	18.7 \pm 0.9	-0.022 \pm 0.002	31.8 \pm 2.5
2.5	15.3 \pm 0.5	16.8 \pm 0.2	-0.025 \pm 0.003	28.2 \pm 3.7
7.5	18.5 \pm 5.8	16.1 \pm 1.4	-0.024 \pm 0.006	30.9 \pm 7.8

When all experiments of nitrate and phosphate enrichment were pooled, a strong linear relationship was found between the Cd AEs in *L. plumulosus* and the Cd distribution in algal cytoplasm (Figure 8). An increased proportion of Cd in the algal cytoplasm compartment possibly led to a significant enhancement of Cd assimilation efficiency in *L. plumulosus*.

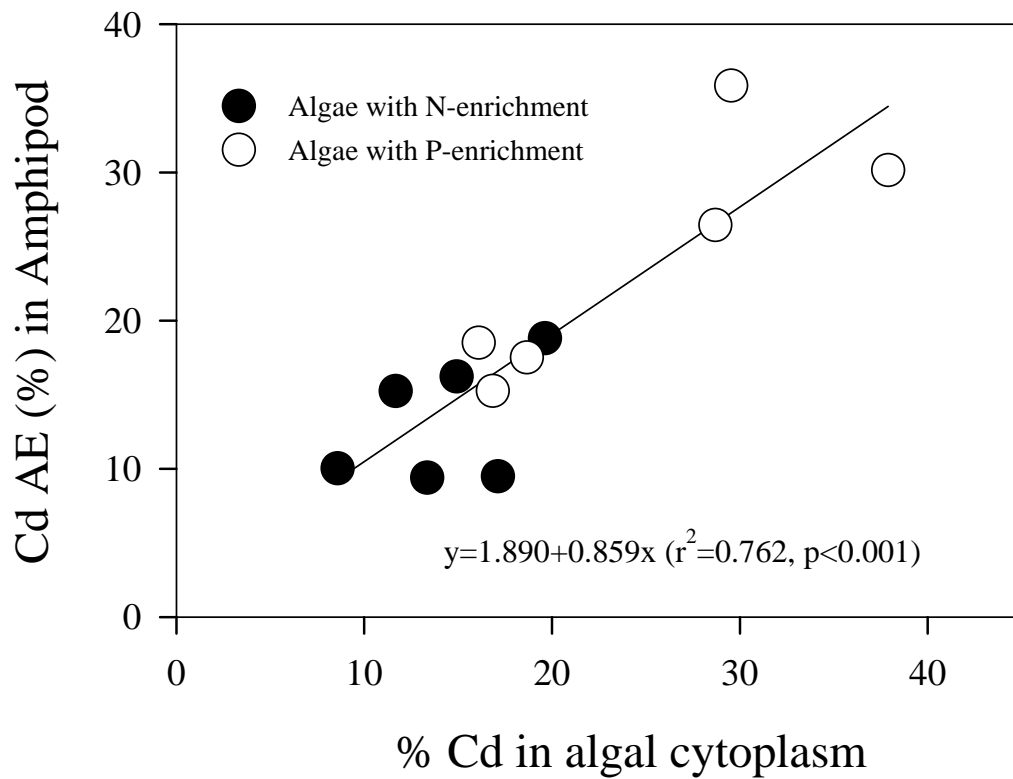


Figure 8. The relationship between Cd distribution in the cytoplasm of algal cells and Cd assimilation efficiency (AE) in *Leptocheirus plumulosus* after feeding on radio-labeled *T. weissflogii*, *N. punctata*, and *I. galbana* grown under different nitrate or phosphate conditions. Data points represent means and standard errors.

DISCUSSION

Assimilation efficiencies of Cd by *L. plumulosus* in this study were in the range of 5.9-18.8% for the planktonic diatom *T. weissflogii*, 10.0-38.8% for the benthic diatom *N. punctata*, and 10.0-16.2% for the planktonic dinoflagellate *I. galbana*. Cell density of *T. weissflogii* used for the pulse-chase feeding in the initial algal species experiment was slightly higher (e.g., 1.0×10^5 cells ml^{-1}) than that used in later experiments (e.g., 2.0×10^4 cells ml^{-1}), thus causing a lower Cd AE of 5.9% in the low end of the range. Higher algal density employed in the radioactive feeding usually yields a lower metal AE (Xu and Wang, 2001; Yu and Wang., 2002). Overall, the Cd AEs in our studies were comparable or slightly higher than those in previous studies with *L. plumulosus*. Schlek et al. (2000) recorded that Cd assimilation efficiencies in *L. plumulosus* were in the range of 2.9-6.2% when fed *Dunaliella tertiolecta*, and 10.6-12.3% when fed *Phaeodactylum tricornutum*. The highest Cd AE (e.g., 26.8%) was found when *L. plumulosus* was fed bacterial exopolymeric coatings. However, in contrast with high Cd AEs in marine copepods (e.g., >90%, Wang et al., 1996) and bivalves (e.g., 37-83%, Reinfelder et al., 1997) after feeding on phytoplankton, the bioavailability of Cd in *L. plumulosus* was generally low.

The higher Cd AEs in the present study compared to those from Schlek et al. (2000) was possibly due to the modified experimental methods employed. In previous studies (Schlek et al., 2000; Schelek et al., 2002), sediment was present in the experimental chambers during the pulse-feeding and depuration period. In our studies, however, only filtered seawater was used in order to simplify possible confounding factors (such as sediment particles) and to easily collect animal feces. A longer depuration duration of 96 h was also employed to measure Cd AE by applying the y-

intercept calculation method, whereas a shorter depuration period was chosen in previous studies (e.g., 4 h, Schlekot et al., 2000). Methods with filtered seawater-only have been broadly used with other invertebrates such as marine mussels (Griscom et al., 2002) and copepods (Wang et al., 1996). *L. plumulosus* performs both surface-deposit and suspension feeding, and amphipods actively fed in and acclimated to seawater-only conditions quickly in our experiments. It was reported that acute 96-h LC₅₀ value of water-only Cd was 300 µg L⁻¹ for juveniles (Schlekot et al., 1992) and 280 µg L⁻¹ for neonates of *L. plumulosus* (DeWitt et al., 1996). The typical Cd background concentration of body burden in another amphipod, *Orchestia gammarellus* (Pallas), ranged from 7.4-12.7 µg g⁻¹ dry wt (Rainbow et al., 1989; Rainbow et al., 1999). Because our experiments only used a background Cd concentration (e.g., 4.12 µg L⁻¹) for algal labeling, no obviously toxic effects (i.e. mortality) were detected, although Cd body burden in *L. plumulosus* was not measured in this study.

Cd Assimilation Influenced by Algal Species, Depuration Substrates and Body Size

Algal diet (i.e. species) significantly affected the assimilation and physiological turnover rate constant (regeneration rate constant) of Cd in *L. plumulosus*. Cd AE from benthic alga *N. punctata* was 6.5× higher than that from *T. weissflogii*, and 2.5× higher than that from *I. galbana*. Similar significant differences of Se AE due to algal diet were also observed in *L. plumulosus* (Schlekot et al., 2002). However, in marine neritic copepods, there were no major influences of different algal species on assimilation of five trace elements (Am, Cd, Co, Se and Zn) (Wang et al., 1996). In this study, food concentrations of the three algae employed for pulse-feeding (e.g., 1×10⁵ cells ml⁻¹ for *T. weissflogii*, 2×10⁵ cells ml⁻¹ for *N. punctata* and *I. galbana*) were relatively comparable

based on algal cell dry weight. However, carbon content in the algae was not determined. Few studies have used benthic algae for metal AE determination because few benthic species grow readily in culture. Benthic diatoms were showed to comprise a major part of diet in one intertidal mudflat amphipod, *Corophium volutator* (Pallas) (Gerdol and Hughes, 1994). These present experiments indicated that Cd bioavailability from the benthic alga *N. punctata* was higher than that from two planktonic algae, possibly indicating that dietary factors (e.g., food preference) might affect Cd assimilation in *L. plumulosus*. Sediment may have high and persistent levels of metal pollution. Higher Cd assimilation from benthic algae to *L. plumulosus* might signify that there is a higher possibility for Cd trophic transfer from benthic algae to grazer invertebrates. Future studies are needed to confirm this result. Most ingested Cd was egested within 24 h in *L. plumulosus*. A similar egestion pattern was also documented in marine bivalves (Chong and Wang, 2000) and freshwater zebra mussels (Roditi and Fisher, 1999). Rainbow (2002) stated that amphipods generally accumulate dietary metals (such as Zn, Cu and Cd) in the ventral caeca of the midgut. Metals are possibly detoxified by forming metal-rich granules in the ventral caeca. Metal-rich granules may be released when epithelial cells break down at the end of cell cycle, resulting in a decrease in the metal concentrations of amphipods and an increase in the metal concentration in fecal pellets (Burgos and Rainbow, 1998).

Depuration in seawater-only of *L. plumulosus* after feeding on labeled algae significantly increased Cd assimilation efficiencies compared with the depuration in natural or processed sediment. The Cd AE in seawater-only was 6.6× higher than in natural sediment and 8.1× higher than in processed sediment. The major variation of Cd

elimination among these three substrates occurred only in the first initial depuration compartment. Approximately 90-95% of Cd was eliminated from amphipod bodies for the natural or processed sediment treatment from 12-24 h, whereas only 60-70% of Cd was released by the seawater-only group during this depuration period. Schlegel et al. (2000) noted that *L. plumulosus* could ingest up to three times their body weight in sediment per day. Rapid and continual ingestion on natural or processed sediment for the amphipods obviously increased the rate of their gut clearance and thus possibly reduced the gut passage time of algae. Lower residence time of metals in the gut might greatly reduce the efficiency of digestion-absorption especially in intracellular digestion of metals, finally resulting in a lower metal AE in animals (Wang and Fisher, 1996; Roditi and Fisher, 1999). In the marine benthic copepod *Coullana* sp., Lotufo (1998) also recorded that depuration of fluoranthene from the animals in sediment (with the elimination rate constant K_d of 0.17 h^{-1}) was significantly faster than in water (with K_d of 0.09 h^{-1}). However, no significant impact on the physiological turnover rate constants (k) among the three depuration substrates ($p = 0.465$) were found in the present study, indicating the regeneration of Cd from *L. plumulosus* in the second depuration compartment was fairly similar.

Although individuals of different body sizes of *L. plumulosus* may have different metabolic activities, body sizes did not show a statistically significant influence on Cd AE or physiological turnover rate constant in our experiments. These results may provide some fundamental understanding of metal AE, suggesting that AE measurements may be independent of body size. Lee et al. (1998) also found that physiological turnover rate constants of Cd, Cr and Zn were not affected by body size in the clam, *Potamocorbula*

amurensis, but body mass in another bivalve *Macoma balthica* did affect the constant for Cd. No previous data for *L. plumulosus* are available for comparison.

Nutrient Enrichment Effects on Cd Trophic Transfer

Nitrate addition from 0 μM (N starvation)-180 μM significantly increased Cd AE in *L. plumulosus* by 2.0 \times for *T. weissflogii*, by 2.7 \times for *N. punctata*, and by 1.6 \times for *I. galbana*. Algal fractionation demonstrated that nitrate enrichment caused significant increase of Cd distribution in algal cytoplasm. There was a significant correlation between Cd distribution in algal cytoplasm and Cd AE in *L. plumulosus*. Reinfelder and Fisher (1991) revealed that assimilation efficiencies of metals in marine copepods were directly related to the cytoplasm content of planktonic diatoms. Recent studies showed that nitrate enrichment also caused a 2.4 \times increase in Cd AE of marine copepod *Calanus sinicus* after nitrate concentration was increased from 17.6-176 μM for a semi-continuous culture of *T. weissflogii* (Wang et al., 2001b). Similar enhanced assimilation of Zn (Wang et al., 2001a) and Fe (Wang and Dei, 2001b) have been observed in *Calanus sinicus* feeding labeled diatoms grown under enhanced nitrate conditions. Considerable increase of algal Cd uptake rates (1.5-4.1 \times) and Cd distribution in algal cytoplasm under nitrate enrichment were associated with this enhancement of Cd trophic transfer in copepods, although no apparent rise of Zn distribution in diatom cytoplasm was reported (Wang et al., 2001a,b). Our experiments further confirmed that ambient nitrate enrichment in algae could significantly increase trophic transfer of Cd in a benthic food chain from algae to *L. plumulosus*. The increased Cd bioavailability in *L. plumulosus* under nitrate addition was presumably due to elevated cytoplasmic Cd distribution in algae. It is believed that Cd is the most effective inducer of phytochelatin production (Ahner and Morel, 1995) and can

also increase the activity of carbonic anhydrase in marine diatoms (Cullen et al., 1999). By increasing the synthesis of these protein-ligands in algae, nitrate enrichment may strongly stimulate Cd uptake and accumulation in algal cytoplasm (Wang et al., 2001b). Furthermore, Cd in the algal cytoplasmic pool is generally regarded as the most bioavailable form for herbivores, which results in a higher Cd AE in grazers.

By pulse-feeding labeled algae and then depurating *L. plumulosus* in chambers with sediment, however, no significant relationship was observed between the distribution of metals in algal cell cytoplasm and AEs for Cd and Ag (Schlekat et al., 2000) or for Se (Schlekat et al., 2002). These results were quite different from ours. Several factors may possibly cause this difference. In the pulse-feeding process as an active surface deposit feeder burrowing in sediment, *L. plumulosus* might keep feeding on both sediment particles and labeled algae with little particle selection. Rapid feeding on particles mixed with radioactive algae possibly not only reduces the gut passage time but also may alter cell density of ingested algae. Our studies showed that depuration in sediment greatly reduced Cd AE compared with depuration in seawater-only (Figure 4). Metal AEs are highly particle dependent (Schlekat et al., 2000), and possibly determined by several important parameters such as metal concentration in ingested particles, ingestion rate, gut passage time and efflux rate (Wang and Fisher, 1999). Therefore, it is possible that different substrates (e.g., sediment) in pulse-feeding and depuration greatly affected metal AE and further changed the correlation between metal AE and cytoplasm distribution of metals in algae.

Nitrate enrichment did not significantly influence physiological turnover rate constants (k) of Cd in *L. plumulosus*. Similarly, no nitrate effects on k values for Cd and

Zn were observed in marine copepod *Calanus sinicus* after feeding on *T. weissflogii* (Wang et al., 2001a,b). The physiological turnover rate constants for Cd in *L. plumulosus* (0.016-0.025 h⁻¹, or 0.384-0.600 d⁻¹) were slightly lower than k values of 0.630-0.668 d⁻¹ in marine copepod *Calanus sinicus* (Wang et al., 2001b), but far higher than values (0.01-0.05 d⁻¹) in marine bivalves (Reinfelder et al., 1997).

No significant effects of phosphate addition on Cd trophic transfer and physiological turnover rate constant in *L. plumulosus* were found. Previous studies indicated that an increase of phosphate concentration from 0-7.2 µM did not significantly influence uptake rate and/or intracellular distribution for both Cd and Zn in marine diatoms (Wang and Dei, 2001a; Wang et al., 2001a). These results might explain the independent causal relationship of Cd trophic transfer from algae to *L. plumulosus* under phosphate enrichment. The generally higher Cd AEs in phosphate experiments than in nitrate trials were possibly caused by the high unmanipulated nitrate levels in P treatments (e.g., 882.4 µM nitrate in the f/2 medium level). Because amphipods such as *L. plumulosus* serve as important surface deposit or suspension feeders in estuarine benthic ecosystems, further studies of nutrient effects on trophic transfer of metals with natural sediment especially in the field are warranted.

SUMMARY

The present study was the first to investigate Cd egestion rate, assimilation efficiency, and trophic transfer influenced by nutrients in *L. plumulosus* in seawater-only environments and the first to determine AEs from benthic microalgae. For benthic amphipods such as *L. plumulosus* burrowing in coastal surface sediment, quantifying metal release from feces is the key point to determine metal cycling in benthic food chains, which is difficult to do with the presence of sediment substrates. Trophic transfer of Cd from microalgae to this amphipod was significantly influenced by algal species in its diet and depuration substrates, but not by body size. Interaction between eutrophication and metal contamination broadly occurs in most coastal areas and wetlands, and sediment contamination may be high and is persistent. This study has shown that nitrate enrichment at environmental realistic levels significantly increased Cd distribution in algal cytoplasm and thus enhanced Cd assimilation efficiencies in *L. plumulosus*, whereas phosphate addition did not. A highly significant correlation was observed between Cd AE in amphipods and cytoplasmic Cd distribution in the feeding algae, which was different from the previous studies. These experiments highlight the important implications that the trophic transfer of metals in benthic ecosystems might be significantly influenced by coastal eutrophication.

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